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Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors

(erythropoietin receptor/phosphoinositide 3-kinase/mitogen-activated protein kinase/Shc/Grb2)

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ABSTRACT **Homodimerization of the erythropoietin (EPO) receptor (EPO-R) in response to EPO binding transiently activates the receptor-associated protein tyrosine kinase JAK2. Tyrosine phosphorylation of the EPO-R creates "docking sites" for SH2 domain(s) in signaling molecules such as the protein tyrosine phosphatases SH-PTP1 and SH-PTP2, phosphoinositide 3-kinase (PI3 kinase), and STAT5. However, little is known about the specific intracellular signals essential for proliferation and differentiation of erythroid progenitors. Here we show that an EPO-R containing only one cytosolic (phospho)tyrosine residue, Y479, induces a signal transduction pathway sufficient for proliferation and differentiation of fetal liver progenitors of erythroid colony-forming units from EPO-R$^{-/-}$ mice as well as for proliferation of cultured hematopoietic cells. This cascade involves sequential EPO-induced recruitment of PI3 kinase to the EPO-R and activation of mitogen-activated protein kinase activity, independent of the Shc/Grb2-adapter pathway and of STAT5. Protein kinase Cε may be one of the mediators connecting PI3 kinase with the mitogen-activated protein kinase signaling cascade. Our results identify a signaling cascade important *in vivo* for erythroid cell proliferation and differentiation.**

Erythropoietin (EPO), a hormone synthesized by the kidney, is the primary regulator of mammalian erythropoiesis. Signaling through the EPO receptor (EPO-R) (1), which is a member of the cytokine receptor family, regulates proliferation, differentiation and survival of erythroid progenitor cells. Homodimerization of the receptor in response to EPO binding (2, 3) transiently activates the receptor-associated protein tyrosine kinase JAK2 (4). Tyrosine phosphorylation of the EPO-R creates "docking sites" for SH2 domain(s) in signaling molecules such as the protein tyrosine phosphatases SH-PTP1 (5) and SH-PTP-2 (6), phosphoinositide 3-kinase (PI3 kinase) (7), and STAT5 (8–10). However, little is known about the specific intracellular signals essential for proliferation and differentiation of erythroid progenitors.

In a number of growth factor receptor systems recruitment of PI3 kinase correlates with mitogenic signaling (11). This lipid kinase is a heterodimeric enzyme composed of the regulatory p85 subunit and the catalytic p110 subunit that phosphorylates phosphoinositides at the D3 position of the inositol ring. Stimulation by many cytokines including EPO (7, 12, 13) results in a transient increase in PI3 kinase activity. Mediated by the SH2-domains of p85, recruitment of PI3 kinase to specific phosphotyrosine residues within the cytoplasmic domain of receptors places the enzyme in close proximity to substrates. Recent evidence suggests that the lipid products act as second messengers and directly activate enzymes such as Akt (protein kinase B) (14) and certain isoforms of protein kinase C (PKC) (15, 16). Proposed downstream targets of PI3 kinase include p70 S6 kinase and MEK [mitogen-activated protein (MAP) kinase (MAPK) kinase]. The latter would represent a novel pathway for activation of the MAPK signaling cascade, independent of the ras/raf activation pathway (17).

Activation of the EPO-R results in tyrosine phosphorylation of Shc (18–20). In other receptor systems tyrosine-phosphorylated Shc forms a complex with the adapter protein Grb2 which in turn is constitutively associated with the guanine-nucleotide exchange protein Son of Sevenless (21). Son of Sevenless-catalyzed exchange of GDP for GTP on Ras results in activation of Ras. Ras-mediated membrane translocation of the serine–threonine kinase Raf results in increased Raf-kinase activity and the initiation of a protein kinase cascade culminating in the activation of MAPK (22).

Here we use gene transfer by retroviral infection of fetal liver cells from EPO-R$^{-/-}$ mice (23) to test the ability of mutant EPO-Rs to support proliferation and differentiation of primary erythroid colony-forming unit (CFU-E) progenitor cells. We show that an EPO-R in which all eight cytosolic tyrosines except Y479 are mutated to phenylalanine induces a signal transduction pathway sufficient for erythroid differentiation as well as for proliferation of cultured cells. This cascade involves sequential EPO-induced recruitment of PI3 kinase to the EPO-R and activation of MAPK activity, and is independent of STAT5. Our results identify a signaling cascade involving activation of MAPK that is important *in vivo* for erythroid cell proliferation and differentiation.

MATERIALS AND METHODS

Plasmids and Generation of Mutant EPO-Rs. The EPO-R and mutant EPO-R cDNAs were generated as described (9) and introduced into the *Bam*HI and *Eco*RI restriction sites of the retrovirus expression vector pBabe (puro) (24). F8 designates a mutant EPO-R lacking all eight tyrosine residues in the cytoplasmic domain. F7Y479 corresponds to a mutant EPO-R

Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; PI3 kinase, phosphoinositide 3-kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK kinase; GST, glutathione *S*-transferase; MBP, myelin basic protein; CFU-E, colony-forming unit, erythroid; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ERK, extracellular signal-regulated kinase.

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in which seven tyrosine residues except for Tyr-479 have been replaced by phenylalanine. Mutant EPO-R Y479F contains the single Y479F point mutation and is described in ref. 9. Other point mutant EPO-Rs in which single tyrosine residues were mutated to phenylalanine were generated by PCR mutagenesis and introduced into the eukaryotic expression vector pXM as described (5).

Retroviral Infection of Fetal Liver Cells and Selection of Stable Ba/F3 Cell Lines. pBabe (puro) clones obtained were transfected by the Ca-phosphate method into the packaging cell line Bosc 23 (25). For each experiment equal expression levels of the various EPO-Rs in the packaging cell lines were confirmed by immunoblotting with anti-EPO-R antiserum to control for equivalent titers of the EPO-R viruses. Twenty-four hours after transfection the medium was changed to Iscove's modified Dulbecco's medium, and 24 hr later 1 ml of virus-containing supernatant was incubated with 10^4 nucleated fetal livers cells from EPO-R$^{-/-}$ mice (23) for 4 hr at 4°C in the presence of 4 μg/ml Polybrene. After centrifugation, cells were plated in methylcellulose supplemented with 3 units EPO/ml and 100 ng/ml stem cell factor. CFU-E colony formation was monitored after 2–3 days and the benzidine-positive colonies were counted.

BaF3 cell pools expressing the wild-type EPO-R or the F8 or F7Y479 EPO-R mutants in the context of pBabe (puro) were generated and characterized as described (9); cell pools expressing the EPO-R in conjunction with the puromycin resistance gene were selected and maintained in the presence of 1.5 μg/ml puromycin (Sigma) and 10% WEHI-conditioned medium as a source of interleukin 3. Cell pools expressing the Y to F mutant EPO-Rs were generated by electroporation and were selected in the presence of 0.6 mg/ml G418 and 10% WEHI-conditioned medium (5). For each of the cell pools used, equivalent cell surface expression of the EPO-Rs ($\pm$50%) was validated by immunoblotting with an anti-EPO-R antiserum and confirmed by cell surface binding experiments with ^{125}I-labeled EPO (data not shown). For growth assays, cells were washed three times and plated at a density of 10^4 cells/well in a 24-well cluster plate in the presence of the indicated EPO concentrations. After three days cell numbers were determined using a Coulter counter.

Immunoprecipitation and Immunoblotting. BaF3 cells expressing the wild type EPO-R or mutant EPO-Rs were starved for 4 hr in serum-free medium in the presence of 1 mg/ml BSA, stimulated for 5 min with EPO 100 unit/ml (provided by Amgen) or 15 min with 200 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) at 37°C, and lysed by addition of an equal volume of 2× lysis buffer as described (5). Pretreatment of cells with 100 nM wortmannin (Sigma) was performed for 30 min in serum-free medium. For each immunoprecipitation lysates corresponding to 10^7 cells were used. Immunoprecipitation experiments were carried out as described (5); the antisera used were: $\approx$1 μg of affinity-purified antiserum directed against the extracellular domain of the murine EPO-R (5); 1 μl of polyclonal rabbit serum recognizing p85 (Upstate Biotechnology), and 5 μg of polyclonal rabbit serum directed against Shc (Transduction Laboratories, Lexington, KY). Analysis was performed by low-bis 15% SDS/PAGE, followed by immunoblotting with the monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology) at a dilution of 1:5000 or an anti-Grb2 serum (Santa Cruz Biotechnology) and enhanced chemiluminescence (DuPont/NEN). For reprobing blots to control for equal loading, they were treated with 2-mercaptoethanol and SDS (5) and incubated with antibodies as indicated.

MAPK Assay. MAPK activity was detected by immunoprecipitation using 0.5 μg of anti-ERK2 antibody (Santa Cruz Biotechnology) followed by an *in vitro* kinase assay on the immunocomplex conducted for 20 min at room temperature in the presence of 0.2 mg/ml myelin basic protein (MBP) and 10 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP in kinase buffer (50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/500 mM NaCl/1 mg/ml BSA). The reaction was terminated by the addition of sample buffer, loaded on a 15% low-bis SDS/PAGE and detected by autoradiography.

Used for transient expression of a glutathione *S*-transferase (GST)-MAPK fusion protein in the experiment depicted in Fig. 2 was the expression vector pcDNA3/GST-extracellular signal-regulated kinase (ERK)1, a kind gift from L. I. Zon (Harvard Medical School, Boston). Also used was pN110, encoding a dominant negative form of PI3 kinase. It was constructed by amplifying a fragment spanning the N-terminal 143 amino acids of the bovine p110 α subunit. The 5′ primer used included a *Hin*dIII restriction site followed by eight amino acids of the FLAG-epitope (Kodak/IBI) fused to the coding region of p110, while the 3′ primer introduced a *Xba*I restriction site. The fragment obtained by PCR-amplification was introduced into the *Hin*dIII and *Xba*I restriction sites of pcDNA3 (Invitrogen), generating pN110. The DNA sequence of the insert was confirmed by sequencing. For transient transfection 50 μg of pN110 or pcDNA3 was introduced together with 2 μg of pcDNA3/GST-ERK1 into BaF3 cells by electroporation as previously described (26). The cells were incubated in normal growth medium (RPMI medium 1640/10% FCS/10% WEHI) for 24 hr and then analyzed for MAPK activity.

RESULTS

Tyr-479 in the EPO-R Can Support Mitogenic Signaling in Cell Lines. To identify the role of the tyrosine residue, Y479, closest to the C terminus of the EPO-R, we introduced the wild-type and several mutant EPO-Rs into the interleukin 3-dependent proB-cell line BaF3. Fig. 1*A* shows that EPO-supported proliferation of BaF3 cells can be dependent on Y479. Expression of the wild-type EPO-R allowed the cells to proliferate in normal concentrations of EPO, while mutant EPO-R F8, lacking all eight tyrosine residues in the cytoplasmic domain, was unable to support EPO-mediated cell proliferation. Importantly, mutant EPO-R F7Y479, lacking all tyrosine residues except for Y479, supported EPO-dependent cell proliferation. Although cells expressing the wild-type EPO-R and EPO-R F7Y479 expressed the same number of cell surface receptors (1,500 per cell, data not shown), cells expressing EPO-R F7Y479 required $\approx$5-fold higher concentration of EPO for proliferation. This observation is consistent with the finding that expression of other mutant EPO-Rs containing single tyrosine residues also suffices for EPO-dependent proliferation of BaF3 cells (27). Therefore, multiple tyrosine residues in the EPO-R can support cell growth; in the wild-type receptor the signals emanating from these tyrosines presumably interact to generate an appropriate level of cell proliferation. However, the signal emanating from EPO-R (phospho)Y479 is one of the most efficient in mediating cell proliferation (27).

Differentiation and Proliferation of Erythroid Progenitors Is Promoted by Tyr-479 in the EPO-R. Fetal livers from embryos homozygous for a deletion of the EPO-R (EPO-R$^{-/-}$) contain erythroid progenitors which undergo terminal differentiation in a standard *in vitro* culture when the wild-type EPO-R is provided by retrovirus infection and both EPO and stem cell factor is added (23, 27). Fig. 1*B* shows that expression of the mutant EPO-R F8 in EPO-R$^{-/-}$ fetal liver cells supports only very low levels of CFU-E colonies, suggesting that tyrosine residues in the cytoplasmic domain of the EPO-R are essential for erythroid differentiation. Importantly, EPO-R F7Y479 supported the formation of >87% of the number of CFU-E colonies, as did the wild-type EPO-R. The size and morphology of these colonies was normal, as was the extent of hemoglobinization as evidenced by the intensity of benzidine

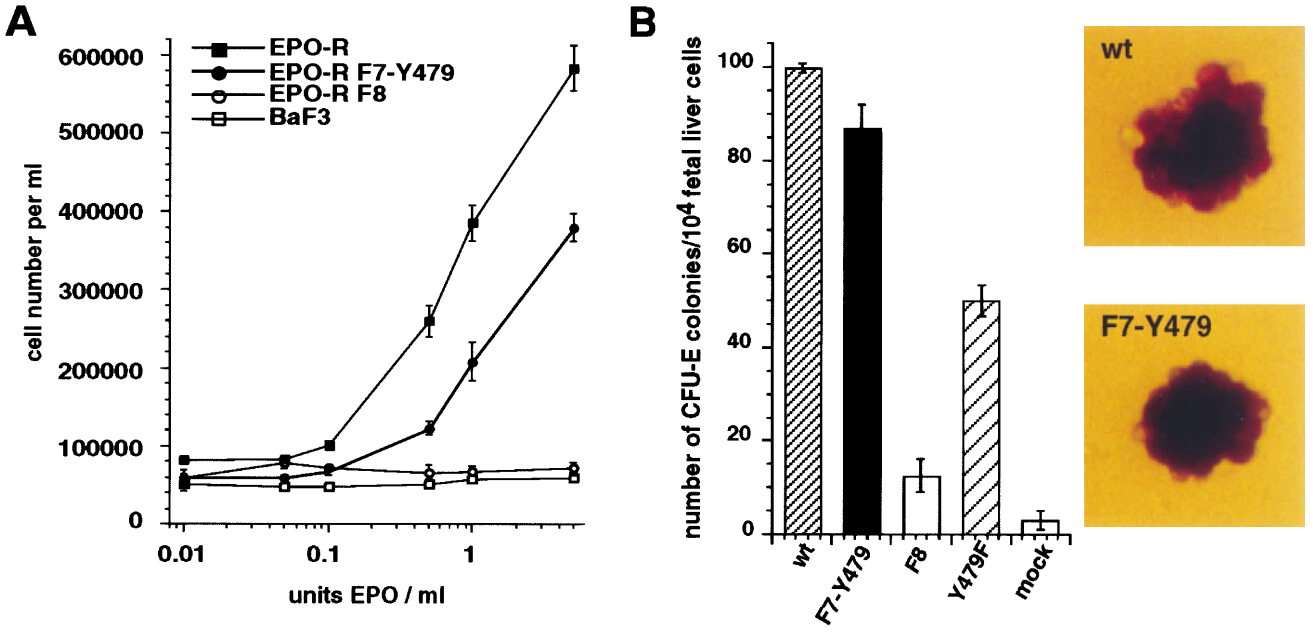

FIG. 1. Tyr-479 in the EPO-R is sufficient to promote EPO-dependent proliferation and differentiation. (*A*) Proliferation of parental BaF3 cells and BaF3 cells expressing the EPO-R or the mutant EPO-Rs F8 or F7Y479 in different concentrations of EPO. The number of cells was determined using a Coulter counter. The average cell numbers per milliliter in four independent determinations (±SD) are plotted against the concentration of EPO (units/ml) added. (*B*) Tyr-479 in the EPO-R is sufficient to support EPO-dependent differentiation of erythroid progenitors. Fetal liver cells harvested from day 12.5 EPO-R$^{-/-}$ mice were infected with retroviruses designed to express either the wild-type EPO-R or the point mutant EPO-Rs F7Y479, F8, or Y479F. The number of benzidine-positive colonies (CFU-Es) formed in the presence of EPO and stem cell factor was counted after 2 days. The results plotted represent the mean (±SD) of three independent experiments. All values are expressed per 10^4 nucleated fetal liver cells. The morphology of CFU-E colonies formed in the presence of EPO and stem cell factor in cultures infected with retroviruses expressing the wild-type EPO-R (wt) or the mutant EPO-R F7Y479 (F7-Y479) is the same.

staining (Fig. 1*B Right*). Thus, a signal capable of supporting normal erythroid proliferation and differentiation emanates from (phospho)Y479. This signal can, at least in part, be generated from other tyrosines in the EPO-R, since mutant EPO-R Y479F (lacking Y479) supported the formation of ≈50% of the number of CFU-E colonies achieved by the wild-type EPO-R (Fig. 1*A*). Since Y479, in a receptor (F7Y479) lacking all other cytosolic tyrosine residues, renders the EPO-R fully competent to promote terminal differentiation of CFU-E progenitors, we conclude that in the EPO-R Y479 represents one of the key residues required *in vivo* for erythroid proliferation and differentiation.

Recruitment of PI3 Kinase Mediates MAPK Activation by the EPO-R. To identify signaling cascades emanating from Y479 we tested the association of mutant EPO-Rs with several signal transduction proteins. As shown in Fig. 2*A* and independently by Damen *et al.* (7), (phospho)Y479 in the EPO-R is the binding site for the p85 subunit of PI3 kinase. The mutant EPO-R Y479F becomes tyrosine phosphorylated after EPO addition yet does not bind to p85 (Fig. 2*A Top* and *Middle*), as judged by coimmunoprecipitation with an antibody specific for the p85 regulatory subunit of PI3 kinase. All other mutant EPO-Rs in which one or two cytosolic tyrosines were changed to phenylalanine bound p85 normally.

The PI3 kinase binding site in the EPO-R-Y479 is important for activation of MAPK activity, since EPO-induced MAPK activation is impaired in cells expressing the mutant EPO-R Y479F, lacking the PI3 kinase binding site (Fig. 2*A Bottom*, lane 8), and is inhibited by pretreatment of cells with the PI3 kinase inhibitor wortmannin (Fig. 2*B*, lane 4). Furthermore, Figs. 2*B* and 3 show a direct correlation between the presence of Y479 in the EPO-R and the ability of the receptor to stimulate MAPK activity. In these experiments MAPK was isolated by immunoprecipitation from cell lines expressing the wild-type or mutant EPO-Rs and subjected to an *in vitro* kinase assay with MBP as exogenous substrate. Upon EPO addition, MAPK activity was stimulated ≈8–10 fold in cell lines expressing the wild-type EPO-R (Fig. 2*B*, lanes 1 and 3; Fig. 3, lanes 1 and 2) and was not increased above basal levels in cells expressing the mutant EPO-R F8, lacking all eight cytosolic tyrosine residues (Fig. 2*B*, lanes 5 and 6). In contrast, cells expressing the mutant EPO-R F7Y479, which contained only the tyrosine that is a crucial part of the PI3 kinase binding site, restored MAPK activation to between 40% (Fig. 2*B*, compare lanes 3 and 8) and 60% (Fig. 3, compare lanes 2 and 8) of the level obtained in cells expressing the wild-type EPO-R.

Additionally, transient expression of a dominant inhibitory form of PI3 kinase, containing only the N terminus of the catalytic p110 subunit, inhibited the activation of MAPK in response to EPO, thus supporting the notion of a direct link between PI3 kinase and the MAPK signaling cascade. As shown in Fig. 2*C*, we transiently transfected a construct encoding a GST-MAPK chimera into BaF3 cells expressing the EPO-R and measured MAPK activity after isolation of the GST-ERK1 protein on glutathione agarose beads. MAPK activity was stimulated 10-fold upon EPO-treatment (Fig. 2*C*, lanes 3 and 4), but inhibited to a 3.3- ± 0.33-fold stimulation by cotransfection of a dominant inhibitory mutant form of PI3 kinase (Fig. 2*C*, lanes 1 and 2).

Activation of MAPK by the EPO-R Does Not Require Maximal EPO-Induced Formation of the Shc/Grb2-Adapter Complex. Since MAPK activation depends predominantly on the presence of the single tyrosine residue in the cytoplasmic domain of the EPO-R that is required for recruitment of PI3 kinase, and since MAPK activation is also blocked by the PI3 kinase inhibitor wortmannin, we hypothesize that activation of the Shc/Grb2-adapter pathway is not sufficient for activation of MAPK mediated by the EPO-R. The experiments in Fig. 4 support this contention. Tyrosine phosphorylation of Shc and complex formation with Grb2 are indicative of activation of the Ras signaling cascade (22, 28). As shown in Fig. 4, EPO-stimulated tyrosine phosphorylation of Shc and association

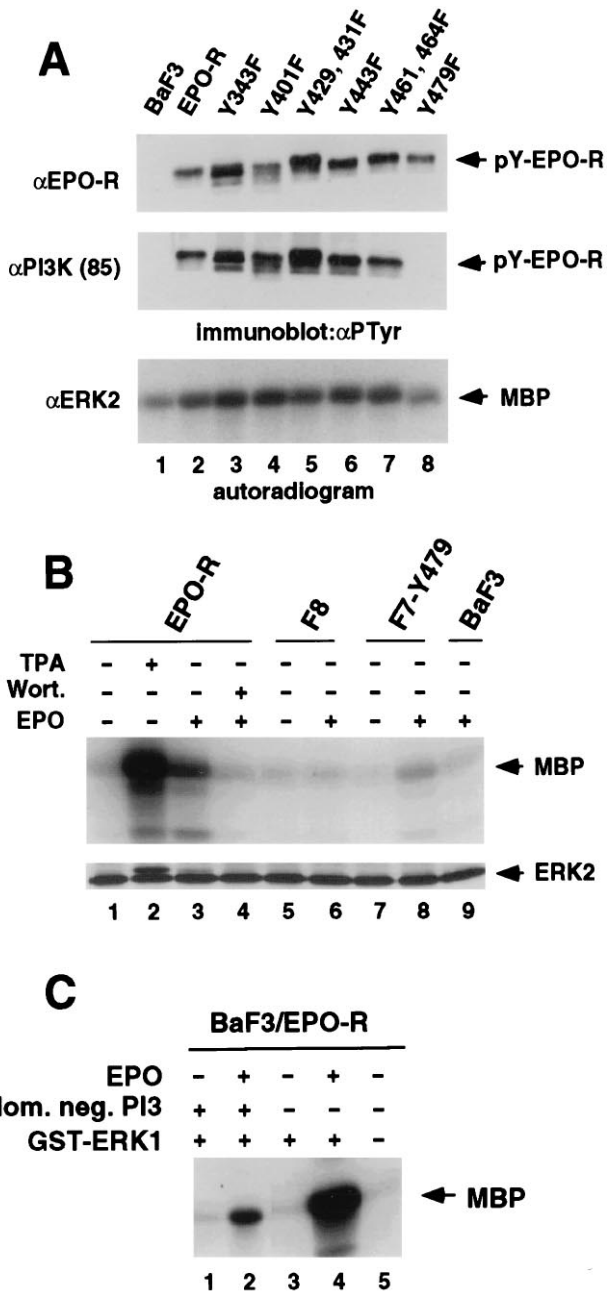

FIG. 2. Tyr-479 is essential for association of the EPO-R with the p85 subunit of PI3 kinase and is important for MAPK activation. (*A*) Parental BaF3 cells and BaF3 cells expressing the wild-type EPO-R or the various mutant EPO-Rs were stimulated with EPO, lysed, and used for immunoprecipitation with antisera directed against the EPO-R, the regulatory subunit of PI3 kinase (p85), or ERK2 as indicated. The tyrosine phosphorylated form of the immunoprecipitated receptor (pY-EPO-R) was detected by immunoblotting with the monoclonal anti-PTyr antibody 4G10 (αPTyr) and is indicated by arrows (*Top* and *Middle*). MAPK immunoprecipitated was subjected to an *in vitro* kinase assay using [γ-^{32}P]ATP. Phosphorylation of the exogenous substrate MBP was detected by autoradiography and is marked by an arrow (*Bottom*). (*B*) Tyr-479 is sufficient for activation of MAPK. Prior to the MAPK assays, parental BaF3 cells and BaF3 cells expressing the wild-type EPO-R or mutant EPO-Rs F8 or F7Y479 were starved as described in *Materials and Methods* and either left unstimulated (−) or treated for 5 min with 100 units EPO per ml (+) prior to lysis. Stimulation for 15 min with 200 nM TPA in the absence of EPO and pre treatment of cells with 100 nM wortmannin in the presence of EPO are indicated (+). MAPK was immunoprecipitated by an antiserum directed against ERK2 and subjected to an *in vitro* kinase assay using [γ-^{32}P]ATP. Phosphorylation of the exogenous substrate MBP was detected by autoradiography and equal immunoprecipitation of the ERK2 MAPK was confirmed by immunoblotting with anti-ERK2. The electrophoretic mobility shift of ERK2 detected in lane 2 and faintly in lanes 3 and 8 indicates the activation of MAPK due to phosphorylation. Similar results (not shown) were obtained when an antibody directed against ERK1 was used for immunoprecipitation. (*C*) An expression vector encoding a GST-ERK1 fusion protein (+) was transiently cotransfected either with an expression vector encoding a dominant negative form of PI3 kinase (dom. neg. PI3) (+) or a control vector into BaF3 cells expressing the EPO-R (BaF3/EPO-R). Twenty-four hours after transfection cells were starved for 4 hr as described and either left unstimulated (−) or treated for 5 min with 100 units EPO per ml (+). The GST-ERK1 fusion protein was precipitated from the lysates by absorption to glutathione agarose beads (Sigma) and subjected to an *in vitro* MAPK assay as described in *Materials and Methods*. Each transfection was done in triplicate and the autoradiogram of one representative experiment is shown.

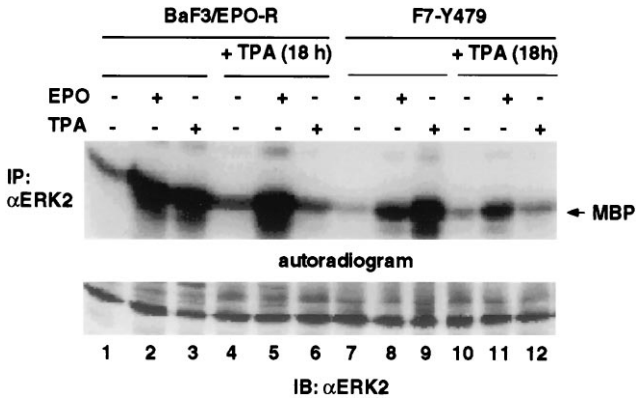

FIG. 3. Stimulation of MAPK activity mediated by the EPO-R involves recruitment of PI3 kinase but does not require maximal activation of PKCα. BaF3 cells expressing the wild-type EPO-R or the mutant EPO-R F7Y479 were either left untreated or were pretreated with TPA as described in *A*, indicated by + TPA (18 h). Prior to lysis cells were left unstimulated (−) or were stimulated either for 5 min with 100 units EPO/ml (+) or for 10 min with 1 μg/ml TPA (+). MAPK activity was immunoprecipitated using an antiserum directed against ERK2 (IP: αERK2) and subjected to an *in vitro* kinase assay in the presence of MBP and [γ-^{32}P]ATP as described. MBP-phosphorylation was detected by analysis on a 15% SDS/PAGE, transfer to a nitrocellulose membrane, and autoradiography (*Upper*). Immunoblotting with an antiserum against ERK2 (IB: αERK2) demonstrates equal loading in all lanes (*Lower*).

with Grb2 does not require the presence of Y479 in the EPO-R; Shc became tyrosine-phosphorylated to the same extent in cells expressing the wild-type EPO-R and the mutant EPO-R Y479F (Fig. 4, lanes 2 and 5). EPO-induced tyrosine phosphorylation of Shc was impaired in cells expressing the mutant EPO-R F8 but was impaired to the same extent in cells expressing EPO-R F7Y479 (Fig. 4, lanes 8 and 11). Possibly the low level of Shc tyrosine phosphorylation in cells expressing EPO-R F8 is mediated by JAK2. In any case, the ability of EPO-R F7Y479 but not EPO-R F8 to trigger erythroid cell proliferation and differentiation (Fig. 1) correlates with its ability to activate the PI3 kinase and MAPK pathways and not with maximal activation of the Shc/Grb2-adapter pathway.

PKCε Is a Possible Mediator Connecting PI3 Kinase with the MAPK Cascade. One possible candidate mediating the effects of PI3 kinase is PKCε, since PKCε is activated *in vitro* and *in vivo* by the lipid products of PI3 kinase, phosphatidylinositol-3,4,5-P_3 and phosphatidylinositol-3,4-P_2 (15, 16). Different PKC isoforms can be distinguished by their sensitivity toward prolonged pretreatment of cells with phorbol esters. To eliminate certain PKC isoforms as candidates, we incubated cells for 18 hr with TPA; in BaF3/EPO-R cells PKCα kinase activity was reduced to almost background levels, while >44% of PKCε kinase activity remained (data not shown). In cells

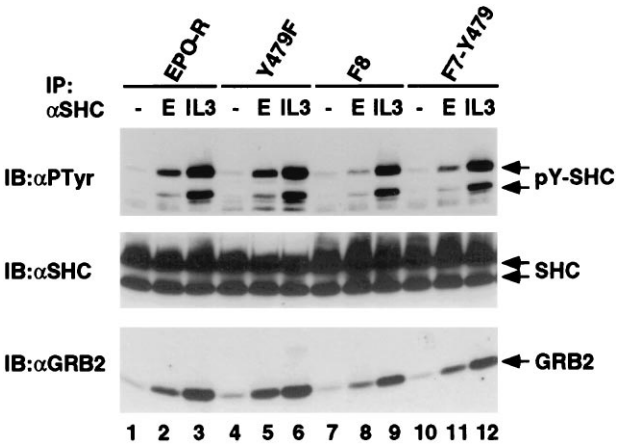

FIG. 4. Stimulation of MAPK activity mediated by the EPO-R does not require maximal activation of the Shc/Grb2-Adapter pathway. After starvation, BaF3 cells expressing the wild-type EPO-R or the mutant EPO-Rs Y479F, F8, or F7Y479 were left unstimulated or stimulated with either 100 units EPO/ml (E) or 1 μg/ml interleukin 3. Lysates were subjected to immunoprecipitation with 5 μg polyclonal rabbit serum directed against Shc. Analysis of immunoprecipitated proteins was conducted by separation on a 15% low-bis SDS/PAGE and successive immunoblotting with anti-PTyr, anti-Shc, and anti-Grb2 antibodies. The positions of tyrosine-phosphorylated Shc (pY-Shc), Shc, and Grb2 are indicated by arrows.

expressing either the wild-type EPO-R or EPO-R F7Y479, short-term treatment with TPA stimulated MAPK activity (Fig. 3, compare lanes 1 and 3, lanes 7 and 9), which was eliminated, as expected, by prolonged TPA-treatment (compare lanes 3 and 6 and 9 and 12). Importantly, prolonged TPA-treatment did not impair EPO-induced MAPK activation (compare lanes 2 and 5 and 8 and 11). Therefore EPO-R-mediated MAPK activation does not require PKCα. This experiment is consistent with a role for PKCε as a link between activation of PI3 kinase by the EPO-R and stimulation of MAPK, but does not exclude other PKC isoforms or other kinases that are activated by phosphatidylinositol-3,4,5-P_3 and that, in turn, activate the MAPK cascade.

DISCUSSION

Tyr-479 Can Support Normal Erythroid Cell Proliferation and Differentiation Here we introduce a unique biological system to study EPO-R signaling. Instead of using cell lines of nonerythroid origin and/or with limited or no erythroid differentiation potential, we took advantage of the fact that mice deficient in EPO or EPO-R accumulate fetal liver CFU-E and BFU-E (burst-forming unit, erythroid) progenitors which cannot differentiate and eventually undergo apoptosis (23, 29, 30). These progenitors will undergo terminal differentiation *in vitro* providing the wild-type EPO-R is reintroduced by retrovirus infection and both EPO and stem cell factor are added (23, 27).

Here, we showed first that a mutant EPO-R lacking all eight cytosolic tyrosine residues is greatly impaired in its ability to support either growth of Ba/F3 hematopoietic cells or differentiation of fetal liver cells from EPO-R$^{-/-}$ mice. This demonstrates that at least one tyrosine residue is essential for proliferation and differentiation in response to EPO. We (27) and others (8, 10) showed that mutation of single tyrosine residues in the EPO-R to phenylalanine did not impair either EPO-induced tyrosine phosphorylation of the receptor or the ability of the receptor to support EPO-mediated proliferation of cell lines. These results indicate that ligand-induced phosphorylation occurs on multiple tyrosine residues of the EPO-R and that redundant intracellular signal transduction pathways mediate proliferation of cell lines in response to EPO.

To identify the role of single tyrosine residues in the EPO-R, in particular the very C-terminal tyrosine residue, Y479, mutant EPO-Rs were generated that contain only one cytosolic tyrosine residue; the remaining seven cytosolic tyrosine residues were mutated to phenylalanine. The presence of only Y479 allowed 70–90% of the cell proliferation obtained by expression of the wild-type EPO-R, but the cells required a $\approx$5-fold higher than normal EPO concentration to grow. We hypothesize that the intracellular signal(s) generated by (phospho)Y479 is sufficient for erythroid proliferation providing the pathway is activated to a greater extent than occurs by the wild-type EPO-R in the presence of the same EPO concentration. That is, in EPO-R F7Y479 only (phospho)Y479 generates a signal for erythroid proliferation and differentiation, and this mutant receptor does stimulate cell growth providing five times the normal number of cell surface receptors are activated by EPO binding.

Importantly, when introduced by retroviral infection into fetal liver cells lacking the wild-type EPO-R, an EPO-R containing only Y479 allowed formation of 90% of the CFU-E colonies obtained with the wild-type EPO-R. Thus, a signal essential for normal erythroid proliferation and differentiation emanates from (phospho)Y479. This signal can, at least in part, be generated from other tyrosines in the EPO-R, since mutant EPO-R Y479F supported the formation of half the number of CFU-E colonies achieved by the wild-type EPO-R. Since Y479, in a receptor (F7Y479) lacking other tyrosine residues renders the EPO-R competent to promote substantial terminal differentiation of CFU-E progenitors, we conclude that in the EPO-R Y479 represents one of the key residues required *in vivo* for erythroid proliferation and differentiation.

The Signal Transduction Pathway Activated by (Phospho)Y479 Involves Sequential Activation of PI3 Kinase and MAPK. In many types of mammalian cells activation of Ras is the key event controlling the activation of the mitogenic signaling cascade involving MAPK (22, 28, 31). However, when activated by cytokine receptors, Ras may serve to prevent apoptosis and not directly stimulate cell proliferation (32, 33). Expression of a dominant negative mutant Ras protein in BaF3 cells eliminated activation of MAPK in response to interleukin 3 (33), suggesting that at least basal levels of Ras activation are required for the stimulation of MAPK in response to cytokines.

Our results show that activation of PI3 kinase by the EPO-R can support activation of MAPK and also erythroid differentiation. This is supported by our finding that pre-treatment of cells with wortmannin blocked the activation of MAPK in response to EPO. A mutant EPO-R (F8) lacking all eight potential tyrosine phosphorylation sites failed to activate MAPK. Tyr-479 is both necessary and sufficient for binding the p85 subunit of PI3 kinase. In particular, EPO-R F7Y479, containing only Y479, the PI3 kinase binding site, supported about half of the level of MAPK activation generated by the wild-type EPO-R.

Traditionally, activation of MAPK has been defined as a linear signal transduction pathway orchestrating the delivery of signals from the plasma membrane to the nucleus. The key element of the traditional MAPK signaling cascade is activation of Ras. EPO-R F7Y479 induced tyrosine phosphorylation of Shc to a level similar to that achieved by mutant EPO-R F8, and also caused formation of the same low amount of a Shc-Grb2 complex as did EPO-R F8. Activation of JAK2, in contrast, is normal in cells expressing EPO-R F8 or EPO-R F7Y479 (9). Since EPO-R F8 cannot activate MAPK, these results show that neither EPO-dependent activation of JAK2, tyrosine phosphorylation of Shc, or formation of a Shc/Grb2 complex is sufficient to fully activate MAPK unless PI3 kinase is also activated. Our study suggests that increases in the lipid

products of PI3 kinase trigger a pathway, perhaps involving activation of PKCε, which, in turn, activates RAF kinase that then activates the MAPK signaling cascade.

Perhaps our most important finding is that activation of MAPK by the EPO-R correlates both with proliferation and differentiation of erythroid progenitors. In the absence of tyrosine residues in the EPO-R, there is a low level of activation of the Shc/Grb2-adapter pathway, possibly mediated by JAK2. Although this pathway may induce membrane translocation of RAF, it is insufficient to support erythroid cell proliferation and differentiation unless a second pathway, independent of STAT5 activation and mediated by (phospho)Y479 in the EPO-R, is activated.

Since a mutant EPO-R lacking the PI3 kinase binding site (Y479F) is able to support formation of some CFU-E colonies, pathways mediated by other tyrosine residues in the EPO-R may be able to substitute, albeit weakly, for the PI3 kinase-MAPK pathway activated by (phospho)Y479. Importantly, our results for the first time identify the PI3 kinase-MAPK pathway as one of the major signals generated by the EPO-R that supports erythroid differentiation *in vivo*. Recently, we showed that redundant signals generated by different tyrosine residues in the EPO-R are sufficient to promote both erythroid cell proliferation and differentiation (27). We propose that different signaling pathways stimulated by the EPO-R are integrated by the MAPK signaling cascade or a protein(s) activated by it, and that the specific outcome presumably depends on the kinetics and magnitude of the activation of the MAPK cascade. However, the intracellular signal transduction proteins activated by the other tyrosine residues in the EPO-R, and their roles in erythroid differentiation, remain to be uncovered.

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